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Protocols

Rapid Isolation of DNA from Dry and Fresh Samples of Plants Producing Large Amounts of Secondary Metabolites and Essential Oils

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Abstract. The presence of certain metabolites have been observed to interfere with DNA isolation procedures and downstream reactions such as DNA restriction, amplification and cloning. The chemotypic heterogeneity among species may not permit optimal DNA yields with a single protocol, and thus, even closely related species may require different isolation protocols. Here we describe the essential steps of a rapid DNA isolation protocol that can be used for diverse medicinal and aromatic plants, which produce essential oils and secondary metabolites such as alkaloids, flavanoids, phenols, gummy polysaccharides, terpenes and quinones. The procedure is applicable to dry as well as fresh plant tissues. This protocol, in our experiments, permitted isolation of DNA from tissues of diverse plant species and produced fairly good yields. The isolated DNA proved amenable to PCR amplification and restriction digestion.

Abbreviations: CTAB, Hexadecyltrimethylammonium bromide.

Key words: DNA isolation, dry samples, essential oil, PCR amplification, secondary metabolites

Introduction

A large number of plant species produce secondary metabolites such as alkaloids, flavanoids, phenols, gummy polysaccharides, terpenes and quinones that are used in food, pharmaceutical, cosmetic and pesticide industries. Many of these medicinal and aromatic plant species are undergoing domestication and cultivar development. In the genetic improvement process, it is desirable to use molecular markers for screening of accessions, choosing of parents

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and selection of progeny. The presence of certain metabolites can hamper the DNA isolation procedures and reactions such as DNA restriction, amplification and cloning. Problems encountered in the isolation and purification of high molecular weight DNA from certain medicinal and aromatic plant species include: (1) degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharides, and (2) inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions (Weishing et al., 1995). The presence of polyphenols, which are powerful oxidising agents present in many plant species, can reduce the yield and purity of extracted DNA (Loomis, 1974; Porebski et al., 1997).

Plant species belonging to the same or related genera can exhibit enormous variability in the complexity of pathways of dispensable functions. Thus, the biochemical composition in plant tissues of different species is expected to vary considerably. The chemotypic heterogeneity among species may not permit optimal DNA yields from one isolation protocol, and perhaps even closely related species may require different isolation protocols (Weishing et al., 1995). Most of the protocols recommend isolation of DNA from fresh tissues, but sometimes the samples collected from remote and rare locations (with the aim of authentication through DNA profiling or solving some intricacies related to molecular taxonomy) may consist of plant parts in dry or semi-dry condition. These situations necessitate the development of the protocols for isolating DNA from different plant organs, including dry tissues. Here we describe a rapid DNA isolation protocol that can be used for diverse medicinal and aromatic plants, using dry as well as fresh plant tissues as the starting material. The protocol permitted isolation of DNA from tissues of diverse plant species in fairly good yields, and the isolated DNA proved amenable to PCR amplification and restriction digestion.

Material and Methods

Reagents and chemicals required

- Tris-Cl pH 8.0 (1.0 M); EDTA pH 8.0 (0.5 M); NaCl (5.0 M); CTAB (20%); Chloroform: Isoamyl alcohol (24:1 v/v); Polyvinylpyrrolidone; and β-mercaptoethanol.
- Extraction buffer: 100 mM Tris-Cl (pH 8.0), 25 mM EDTA, 1.5 M NaCl, 2.5% CTAB, 0.2% β-mercaptoethanol (v/v) (added immediately before use) and 1% PVP (w/v) (added immediately before use).
- High salt TE buffer: 1 M NaCl, 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA.

- Enzymes: *Eco*R I (Amersham Pharmacia) and *Taq* DNA Polymerase (Genei, India).
- Buffers: *Eco*R I buffer (Amersham Pharmacia) and *Taq* DNA polymerase buffer (Genei, India).
- Nucleotides: dNTPs (G, A, T, C) and primers (Genei, India).

Plant samples for DNA isolation

Samples of plant material were collected, in the form of leaves, stem, whole flowers or their parts, from *Allium sativum* (24 accessions), *Artemisia annua* (100 accessions), *Bacopa monnieri* (23 accessions), *Catharanthus roseus* (52 accessions), *Cymbopogon winterianus* (9 accessions), *Pelargonium graveolens* (8 accessions), *Mentha arvensis* (18 accessions), *Ocimum kilmandscharicum* (1 accession), *Taxus wallichiana* (24 accessions) and *Vetiveria zizanioides* (4 accessions). About half of the material from each accession was dried at 50 °C for 48 h and the rest was frozen in liquid nitrogen. DNA was extracted from both fresh and dry samples of each material. The aliquots (2 µl) of DNA from each sample were loaded on a 0.7% agarose gel to check the quality, and about 2 µg of DNA from each preparation was restricted with *Eco*R I according to the guidelines provided by the supplier.

PCR amplification

Polymerase chain reactions (PCRs) for amplification of DNA preparations were carried out in a 25 µl volume. A reaction tube contained 25 ng of DNA, 0.2 units of *Taq* DNA polymerase, 100 µM each of dNTPs, 1.5 mM $MgCl_2$ and 5 pmol of decanucleotide primers. The amplifications were carried out using the DNA Engine thermal cycler (MJ Research, USA) and with the protocol of Khanuja et al. (1998). The amplified products were loaded in a 1.2% agarose gel containing 5 µg ml^{-1} of ethidium bromide and photographed by a polaroid system. Custom decanucleotide primers were designed at CIMAP and were synthesised from M/S Bangalore Genie, India. These were designated MAP01 to MAP12 and had the following sequences respectively: 5′-AAATCGGAGC-3′, 5′-GTCCTACTCG-3′, 5′-GTCCTTAGCG-3′, 5′-TGCGCGATCG-3′, 5′-AACGTACGCG-3′, 5′-GCACGCCGGA-3′, 5′-CACCCTGCGC-3′, 5′-CTATCGCCGC-3′, 5′-CGGGATCCGC-3′, 5′-GCGAATTCCG-3′, 5′-CCCTGCAGGC-3′, and 5′-CCAAGCTTGC-3′.

DNA isolation protocol

- Grind the plant material in liquid nitrogen (3 g fresh tissue or 0.5 g of dry tissue).

- Transfer the material to 10 ml polypropylene tube and add 3 ml of freshly prepared extraction buffer, mix by inversion to a slurry.
- Incubate at 60 °C in a shaking waterbath (100 rpm) for 1–2 h (dry samples may require overnight incubation at 37 °C).
- Add 3 ml of chloroform: isoamylalcohol (24:1) and mix by inversion for about 15 min.
- Spin at 8000 rpm for 10 min at 25–30 °C.
- Carefully transfer the upper clear aqueous layer to another 10 ml polypropylene tube.
- Add 1.5 ml of 5 M NaCl and mix properly (do not vortex).
- Add 0.6 volume of isopropanol and let the mixture stand at room temperature for 1 h. After 1 h, slow and careful mixing will produce fibrous nucleic acid that can be scooped and transferred to a 1.5 ml microfuge tube and centrifuged. Alternatively, after mixing with isopropanol, the samples can be centrifuged at 10,000 rpm for 10 min at 25–30 °C.
- Discard the supernatant and wash the pellet with 80% ethanol.
- Dry the pellet in a vacuum for 15 min and dissolve it in 0.5 ml of high salt TE buffer.
- Add 5 μl of RNase A and incubate at 37 °C for 30 min.
- Extract with equal volume of chloroform: isoamyl alcohol (24:1).
- Transfer the aqueous layer to a fresh 1.5 ml microfuge tube and add 2 volumes of cold ethanol.
- Spin at 10,000 rpm for 10 min at 25–30 °C.
- Wash the pellet with 80% ethanol.
- Dry the pellet in a vacuum and dissolve in 200 μl of sterile double distilled water.
- DNA concentrations can be measured by running aliquots on an 0.8% agarose gel or by taking the absorbance at 260 nm.
- Use about 2 μg DNA for restriction digestion and 25 ng for PCR amplification.

Results and Discussion

The plants that are sources of natural products or bio-active substances also produce large amounts of secondary metabolites and substances of medicinal or industrial importance. Thus, while working with a variety of plants it is common to encounter problems arising from the presence of essential oils, polysaccharides, polyphenols and other secondary metabolites in the lysate and the DNA preparations. The secondary compounds may hamper DNA isolation as well as any further reaction to be carried out on DNA preparations – for example, restriction enzymes may be inhibited because of the

Table 1. Yield of DNA isolated from various samples of different species of medicinal and aromatic plants using the CTAB procedure modified in this study.

Plant (family)	Number of accessions	Organ/tissue	DNA yield μg/g tissue	
			Fresh	Dry
Allium sativum (Alliaceae)	24	Leaf	40	60
Artemisia annua (Asteraceae)	100	Leaf, Florets	20	35
Bacopa monnieri (Scrophulariaceae)	23	Leaf, Stem	25	40
Catharanthus roseus (Apocynaceae)	52	Leaf, Petals	35	50
Cymbopogon winterianus (Poaceae)	9	Leaf	54	80
Pelargonium graveolens (Geraniaceae)	8	Leaf	50	75
Mentha arvensis (Lamiaceae)	18	Leaf	54	85
Ocimum kilmandscharicum (Lamiaceae)	1	Leaf	15	20
Taxus wallichiana (Taxaceae)	24	Leaf	40	55
Vetiveria zizanioides (Poaceae)	4	Leaf	45	65

presence of unusual substances. In our experiments with a variety of medicinal and aromatic plants, we encountered difficulties from the stage of cell lysis to DNA separation in the supernatant and subsequent reactions when following the procedures described by Doyle and Doyle (1987), Murray and Thompson (1980), Dellaporta et al. (1983) and Porebski et al. (1997). Major problems encountered were low DNA yield or poor PCR amplification reactions and restriction endonuclease digestion for hybridisation based DNA fingerprinting and preparation of fragments in cloning reactions. The protocol described by Porebski et al. (1997) yielded more than 20 μg of DNA/g of fresh leaf tissue of *B. monnieri*, *M. arvensis* and *O. kilmandscharicum*; it did not work for dry leaf tissues of any of the plant tissue tried. Also, the procedure suffered from the precipitation of DNA and CTAB left in the supernatant during incubation of the mixture in cold temperatures. Our protocol involves isopropanol precipitation of DNA initially at room temperature. Moreover, the procedure also eliminates the necessity of phenol, which makes the method less hazardous. Further, the addition of high concentration of PVP and β-mercaptoethanol were helpful in removing the polyphenols from *A. sativum*, *A. annua*, *C. roseus*, *C. winterianus*, *P. graveolens*, *T. wallichiana* and *V. zizanioides*. The problem arising from the presence of high levels of polysaccharides was overcome by using NaCl at a higher concentration.

The above protocol invariably achieved good yield of high quality DNA from fresh as well as dry leaf tissues of the above plants, including essential oil producing monocot plants like *A. sativum*, *C. winterianus* and *V. zizan-*

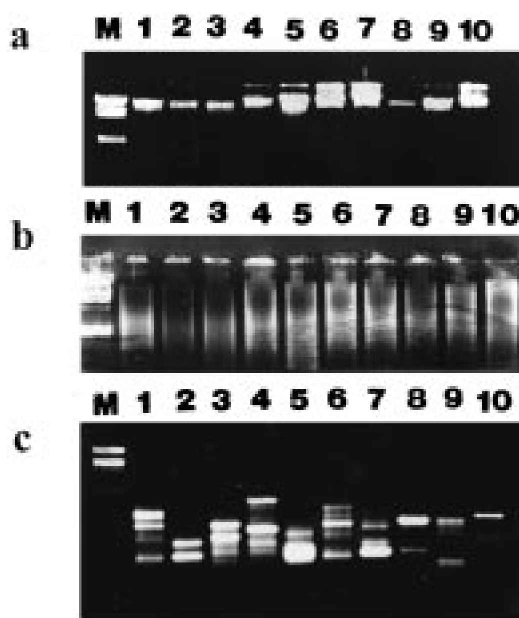

Figure 1. Isolated DNA from ten plant species resolved on 0.8% agarose gel. Figure 1a, purified DNA; figure 1b, DNA samples cut with *Eco*R I; figure 1c, PCR profiles of the DNA samples amplified with primer 5′-GCACGCCGGA-3′. Samples on gel lanes are: M: Marker, λ DNA digested with *Hin*d III; 1, *Allium sativum*; 2, *Artemisia annua*; 3, *Bacopa monnieri*; 4, *Catharanthus roseus*; 5, *Cymbopogon winterianus*; 6, *Pelargonium graveolens*; 7, *Mentha arvensis*; 8, *Ocimum kilmandscharicum*; 9, *Taxus wallichiana*; 10, *Vetiveria zizanioides*.

ioides, and dicot plants like *A. annua*, *P. graveolens*, *M. arvensis* and *O. kilmandscharicum*. The plant, *T. wallichiana*, produces a secondary metabolite taxol and its precursors, which are known to be anticancerous. These compounds, when isolated along with the DNA, do inhibit PCR amplification and restriction digestion. Similarly, in *C. roseus*, compounds like vincristine, and vinblastine inhibit the enzymatic reactions with DNA. The cells of plants like *P. graveolens*, *C. roseus*, *A. sativum*, *C. winterianus* and *V. zizanioides* are known to contain high concentrations of polysaccharides in addition to the active metabolites, complicating the problem of DNA isolation. By using the above protocol a fairly high yield of high quality DNA was obtained for each plant (Figure 1a). The amount of DNA recovered per g of plant material (fresh as well as dry) was sufficiently high and this recovery was possible from various plant parts as well (Table 1). The purity and clean nature of DNA samples could be confirmed through complete digestion by the restriction enzyme *Eco*R I (4 units/μg DNA) after incubating the reaction tubes at 37 °C for 1.5 h (Figure 1b). This indicated that the isolated DNA was amenable to further processing in cloning experiments as well as DNA fingerprinting. The

utility of the isolated DNA for use in PCR amplification for RAPD profiling was demonstrated with several random primers and with DNA preparations of all the plant species tested (Figure 1c). The method described here is, therefore, rapid, simple and efficient for the isolation of DNA from plants that possess a wide range of activities that can interfere with DNA extraction and analysis.

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